

## Characterization of *comQ* and *comX*, Two Genes Required for Production of ComX Pheromone in *Bacillus subtilis*

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Many microbes use secreted peptide-signaling molecules to stimulate changes in gene expression in response to high population density, a process called quorum sensing. ComX pheromone is a modified 10-amino-acid peptide used by *Bacillus subtilis* to modulate changes in gene expression in response to crowding. *comQ* and *comX* are required for production of ComX pheromone. We found that accumulation of ComX pheromone in culture supernatant paralleled cell growth, indicating that there was no autoinduction of production of ComX pheromone. We overexpressed *comQ* and *comX* separately and together and found that overexpression of *comX* alone was sufficient to cause an increase in production of ComX pheromone and early induction of a quorum-responsive promoter. These results indicate that the extracellular concentration of ComX pheromone plays a major role in determining the timing of the quorum response and that expression of *comX* is limiting for production of ComX pheromone. We made alanine substitutions in the residues that comprise the peptide backbone of ComX pheromone. Analysis of these mutants highlighted the importance of the modification for ComX pheromone function and identified three residues (T50, G54, and D55) that are unlikely to interact with proteins involved in production of or response to ComX pheromone. We have also identified and mutated a putative isoprenoid binding domain of ComQ. Mutations in this domain eliminated production of ComX pheromone, consistent with the hypothesis that ComQ is involved in modifying ComX pheromone and that the modification is likely to be an isoprenoid.

Many organisms produce and respond to extracellular signaling molecules in a population density-dependent manner, a process called quorum sensing or the quorum response (7, 9). Quorum sensing regulates a variety of processes, including the production of exoenzymes, genetic exchange, and interactions with eukaryotic hosts, both pathogenic and symbiotic (7).

*Bacillus subtilis* uses at least two peptides, the ComX pheromone (24) and the competence and sporulation factor (CSF) (22, 35), to sense and respond to high population density. Changes in gene expression in response to crowding are mediated by the phosphorylated form of the transcription factor ComA (ComA~P). ComX pheromone and CSF act through convergent signaling pathways to activate the transcription of quorum-responsive genes (genes whose expression is regulated in a density-dependent manner), most likely by increasing the level of ComA~P in cells. ComX pheromone activates the histidine kinase ComP, leading to ComP autophosphorylation and transfer of phosphate to the response regulator ComA (24, 30, 35, 37). CSF is imported by the oligopeptide permease Opp (Spo0K) and acts intracellularly to affect the expression of ComA~P-controlled genes (22). Genetic data indicate that CSF negatively regulates the activity of RapC, a putative ComA~P phosphatase (22, 34). Among the known targets of ComA~P is *srfA*, an operon required for the development of genetic competence in *B. subtilis* (10, 11, 15, 26, 27, 32).

ComX pheromone is a 10-amino-acid peptide (ADPIT RQWGD) with a hydrophobic modification of unknown structure on the tryptophan residue. Two genes, *comQ* and *comX*,

are known to be required for ComX pheromone production (24). *comX* encodes the 55-amino-acid precursor of ComX pheromone. *comQ* is located immediately upstream of *comX* in the chromosome (40), and *comQ* null mutants do not produce ComX pheromone (24). Production of ComX pheromone requires processing of the 55-amino-acid precursor to 10 amino acids, modification of the tryptophan residue, and export from the cell (Fig. 1). ComQ may be involved in the processing and/or modification step (23).

Recently, the sequences of the *comQXPA* regions of a number of *B. subtilis*, *Bacillus mojavensis* and *Bacillus natto* species have been determined (37, 38). While the sequences of the histidine kinase domain of ComP and the response regulator domain of ComA are nearly identical in different *Bacillus* species, the sequences of ComQ and ComX and the N-terminal domain of ComP are more divergent (37, 38). The sequences of ComQ in *B. subtilis* 168 and *B. natto* NAF4 are 44% identical (38). The sequences of the 3' end of *comX*, predicted to encode ComX pheromone, are extremely polymorphic, with the exception of that for the tryptophan residue that is the site of modification in *B. subtilis* 168 (37, 38).

We have characterized various aspects of production of ComX pheromone in *B. subtilis* 168. We found that accumulation of ComX pheromone activity in culture supernatant parallels cell growth, indicating that, in contrast to many of the acyl homoserine lactone signaling systems of gram-negative bacteria, production of this signaling molecule is not autoinduced. We found that overexpression of *comQ* and *comX* or *comX* alone is sufficient to cause induction of quorum-responsive genes (*srfA*) at lower cell density than in wild-type cells. Strains that overexpress *comX* produced more ComX pheromone than wild-type cells, indicating that *comX* is normally

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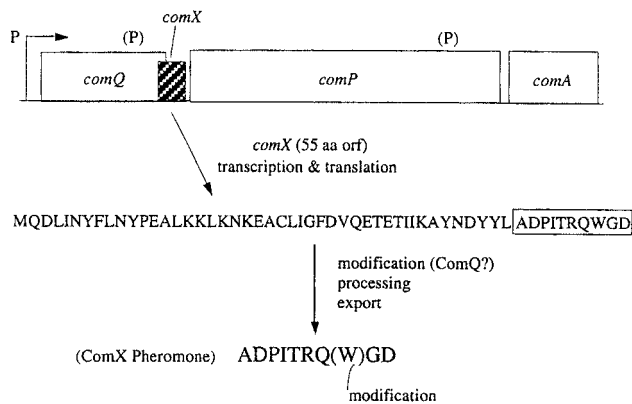


FIG. 1. Production of ComX pheromone. Production of ComX pheromone requires expression of the 55-amino-acid (aa) precursor (encoded by *comX*), modification of the tryptophan at position 53, processing, and export from the cell. The final 10 amino acids, which comprise the peptide backbone of ComX pheromone, are boxed. *comP* and *comA* encode the two-component system required for response to ComX pheromone. Known promoters are labeled with an arrow and a P; putative promoters are indicated by (P). orf, open reading frame.

limiting for ComX pheromone production. Alanine-scanning mutagenesis of the final 9 codons of *comX* highlighted the importance of the tryptophan residue (site of modification) for activity and identified three residues (T50, G54, and D55) that are unlikely to make significant contacts with either the production or response machinery. We found that a putative isoprenoid binding domain in ComQ is required for function in vivo, consistent with the notion that the modification on ComX pheromone is likely to be an isoprenoid.

MATERIALS AND METHODS

**Strains, plasmids, and media.** The *B. subtilis* strains used are listed in Table 1. All strains are derivatives of JH642 (29) and carry the *trpC2* and *pheA1* mutations. Standard procedures were used for transformations and strain construction (13). Plasmids are described in Table 2 and below and were constructed by standard techniques (33).

Routine growth and maintenance of *Escherichia coli* and *B. subtilis* cultures were done in Luria-Bertani (LB) medium. Defined minimal medium containing S7 salts (S750 minimal medium) was prepared as described (39), except that MOPS (morpholinepropanesulfonic acid) buffer was present at 50 mM (15). Minimal medium contained glucose (1%), glutamate (0.1%), and required amino acids (40 µg/ml for tryptophan and phenylalanine, 120 µg/ml for threonine). Antibiotics were used at the following concentrations: ampicillin (Amp) at 100 µg/ml, neomycin (Neo) at 5 µg/ml, chloramphenicol at 5 µg/ml, and erythromycin and lincomycin together (MLS) at 0.5 and 12.5 µg/ml, respectively.

**Overexpression of *comQ* and/or *comX*.** pTH2 was created to overexpress *comQ* and *comX* on a multicopy plasmid. pTH2 contains a 2,679-bp DNA fragment from the *MunI* site of *degQ* (the gene upstream of *comQ*) to the 5'-most *HindIII* site of *comP* (downstream of *comX*) (20) cloned into the *EcoRI* and *HindIII* sites of the *B. subtilis*-*E. coli* shuttle vector pHP13 (13). Thus, pTH2 contains the final 111 bp of *degQ*, the *degQ*-*comQ* intergenic region (including the *comQ* promoter) (40), all of *comQ*, all of *comX*, and the first 1,313 bp of *comP*.

pKB1 was used to make the *comQ* *comX* diploid strains (strains that contained an extra copy of *comQ* and *comX*) and was made by cloning the *BclI*-*StyI* fragment of pTH2 (containing the final 40 bp of *degQ*, the *degQ*-*comQ* intergenic region, all of *comQ*, all of *comX*, and the first 590 bp of *comP*) into pDG795. pKB1 was integrated by double-crossover recombination at the *thrC* locus.

pKB72 was used to make a strain that overexpresses *comX*. *comX* was cloned under the control of the LacI-repressible, IPTG (isopropylthiogalactopyranoside)-inducible promoter Pspac-hy (31) and placed into pDG795 to produce pKB72. pKB72 (Pspac-hy-*comX*) was integrated by double-crossover recombi-

TABLE 1. *B. subtilis* strains used

Strain	Relevant genotype
JMS424	Δ <i>comQ</i> :: <i>spc amyE</i> ::( <i>srfA-lacZ</i> Ω374 <i>neo</i> )
KTB102	<i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) <i>thrC</i> ::( <i>comQ</i> <sup>+</sup> <i>comX</i> <sup>+</sup> <i>mls</i> )
KTB121	<i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) <i>thrC</i> :: <i>mls</i>
KTB122	Δ <i>comQ</i> :: <i>spc thrC</i> :: <i>mls</i>
KTB131	Δ <i>comQ</i> :: <i>spc thrC</i> ::( <i>comQ</i> <sup>+</sup> <i>mls</i> )
KTB172	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pHP13 ( <i>cat mls</i> )
KTB216	Δ <i>comQ</i> :: <i>spc</i> pKB28 ( <i>comQD67E cat mls</i> ) <i>thrC</i> :: <i>mls</i>
KTB217	Δ <i>comQ</i> :: <i>spc</i> pKB29 ( <i>comQD71E cat mls</i> ) <i>thrC</i> :: <i>mls</i>
KTB280	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB58B ( <i>comX</i> <sup>+</sup> <i>cat mls</i> )
KTB282	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB59 ( <i>comXG54A cat mls</i> )
KTB288	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB62 ( <i>comXR51A cat mls</i> )
KTB300	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB64 ( <i>comXP48A cat mls</i> )
KTB302	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB65 ( <i>comX149A cat mls</i> )
KTB304	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB66 ( <i>comXQ52A cat mls</i> )
KTB306	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB67 ( <i>comXD55A cat mls</i> )
KTB308	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB68 ( <i>comXW53A cat mls</i> )
KTB310	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB69 ( <i>comXD47A cat mls</i> )
KTB344	<i>thrC</i> :: <i>mls</i>
KTB345	<i>thrC</i> ::( <i>comQ</i> <sup>+</sup> <i>comX</i> <sup>+</sup> <i>mls</i> )
KTB346	<i>thrC</i> ::(Pspac-hy <i>mls</i> )
KTB348	pKB71 ( <i>comQ</i> <sup>+</sup> <i>cat mls</i> )
KTB349	<i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB71 ( <i>comQ</i> <sup>+</sup> <i>cat mls</i> )
KTB360	<i>thrC</i> ::(Pspac-hy- <i>comX</i> <sup>+</sup> <i>mls</i> )
KTB363	Δ <i>comX</i> <i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pKB78 ( <i>comXT50A cat mls</i> )
TMH142	pTH2 ( <i>comQ</i> <sup>+</sup> <i>comX</i> <sup>+</sup> <i>cat mls</i> )
TMH143	pHP13 ( <i>cat mls</i> )
TMH326	<i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pTH2 ( <i>cat mls</i> )
TMH327	<i>amyE</i> ::( <i>srfA-lacZ</i> Ω682 <i>neo</i> ) pHP13 ( <i>cat mls</i> )

nation at the *thrC* locus. Pspac-hy-*comX*<sup>+</sup> complements a *comX* but not a *comQ* null mutation (data not shown).

To overproduce *comQ* in the absence of *comX*, the *HindIII*-*HindIII* fragment of pTH2 was excised and the vector was religated, creating pKB71. pKB71 contains all of *comQ* (under the control of the native *comQ* promoter) and a truncated version of *comX* that is missing the final 48 bp (and thus the sequence encoding the backbone of ComX pheromone). This plasmid is able to complement a *comQ* null mutation but not a *comX* null mutation (data not shown).

**Construction and expression of ComX alanine mutants.** *comX* alanine mutations were introduced by oligonucleotide-directed mutagenesis (19, 33) using pKB6 (*comX* in pGEMcat [13]) as the template DNA. This created a set of plasmids, each encoding one alanine variant of *comX*. The presence of each mutation was confirmed by DNA sequencing.

Plasmid pKB58 contains the *comQ* promoter and was made by PCR amplifying the region from the 3' end of *degQ* up to but not including the *comQ* ribosome-binding site (using primers KBP31 and KBP32; sequences available upon request) and cloning the PCR product between the *EcoRI* and *SmaI* sites of pHP13. Each *comX* allele was amplified by PCR and cloned into pKB58, creating a set of plasmids (pKB58B, pKB59, pKB62, pKB64-pKB69, and pKB78) in which the *comX* allele is under control of the *comQ* promoter. The presence of the mutations and the integrity of the junctions were confirmed by isolating each plasmid from *B. subtilis*, amplifying *comX* by PCR, and sequencing the PCR products.

**Construction and expression of *comQ* mutant alleles.** The *comQD67E* and *comQD71E* mutant alleles were created by oligonucleotide mutagenesis using pNG15 (24) as the template DNA. The presence of the appropriate *comQ* mutation in pKB23 (*comQD67E* in pGEMcat) and pKB24 (*comQD71E* in pGEMcat) was confirmed by DNA sequencing.

TABLE 2. Plasmids used

Plasmid	Description	Source or reference
pHP13	Multicopy vector; <i>cat erm</i>	13
pDG795	Cloning vector for recombination into <i>thrC</i> ; <i>erm</i>	P. Stragier
pGEMcat	Cloning vector for single-crossover integration	13
pNG15	<i>comQ</i> in pGEMcat; used for site-directed mutagenesis of <i>comQ</i>	24
pTH2	Multicopy plasmid containing <i>comQ</i> and <i>comX</i> ; extends from <i>MunI</i> site in <i>degQ</i> (upstream from <i>comQ</i> ) to first <i>HindIII</i> site in <i>comP</i> (downstream from <i>comX</i> ) cloned into pHP13	This study
pKB1	<i>comQ</i> and <i>comX</i> cloned into pDG795; used to construct <i>comQ comX</i> diploid strains (by recombining <i>comQ</i> and <i>comX</i> into the chromosome at <i>thrC</i> )	This study
pKB6	<i>comX</i> in pGEM-cat; used for site-directed mutagenesis of <i>comX</i>	This study
pKB23	<i>comQD67E</i> made by oligonucleotide-directed mutagenesis of pNG15	This study
pKB24	<i>comQD71E</i> made by oligonucleotide-directed mutagenesis of pNG15	This study
pKB28	<i>comQD67E</i> cloned into pHP13; used to test function of mutant ComQ protein	This study
pKB29	<i>comQD71E</i> cloned into pHP13; used to test function of mutant ComQ protein	This study
pKB58	Contains <i>comQ</i> promoter cloned into pHP13; used to drive transcription of alanine mutants of <i>comX</i>	This study
pKB58B	<i>comX</i> <sup>+</sup> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB59	<i>comXG54A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB62	<i>comXSR51A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB64	<i>comXPR48A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB65	<i>comX149A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB66	<i>comXQ52A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB67	<i>comXD55A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB68	<i>comXW53A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB69	<i>comXD47A</i> transcribed from <i>comQ</i> promoter in pKB58	This study
pKB71	Multicopy plasmid containing <i>comQ</i> <sup>+</sup> (in pHP13)	This study
pKB72	<i>comX</i> <sup>+</sup> under control of the <i>lacI</i> -repressible, IPTG-inducible promoter Pspac-hycloned in pDG795; used to recombine Pspac-hy- <i>comX</i> into the chromosome at <i>thrC</i>	This study
pKB78	<i>comXT50A</i> transcribed from <i>comQ</i> promoter in pKB58	This study

The *BclI*-*HindIII* fragment of pKB23 and pKB24 (containing the final 40 bp of *degQ* through the first 118 bp of *comX*) was cloned between the *Bam*HI and *Hind*III sites of pHP13 to create pKB28 (*comQD67E*) and pKB29 (*comQD71E*), respectively. The *comQD67E* and *comQD71E* alleles did not complement the *comQ* null mutation, as judged from *srfA-lacZ* induction (data not shown).

**β-Galactosidase assays.** β-Galactosidase specific activity was measured essentially as described (15, 24, 34) and is presented as ( $\Delta A_{420}$  per minute per milliliter of culture per unit of optical density at 600 nm [ $OD_{600}$ ])  $\times 1,000$ .

**Fractionation of conditioned medium and bioassay for ComX pheromone.** Conditioned medium was prepared and fractionated and ComX pheromone activity was determined essentially as described (21, 24, 34). Briefly, cells were grown in defined minimal medium, and samples (typically 10 ml) were taken at various times during growth. Cells were removed by centrifugation, and the culture supernatant (conditioned medium) was filtered through a 0.45-μm filter and stored at 4°C until fractionated.

Conditioned medium was fractionated essentially as described (21). A sample (typically 10 ml) of conditioned medium was brought to pH 2 with trifluoroacetic acid (TFA) and loaded onto a 1-ml C<sub>18</sub> reverse-phase column (SepPak; Waters) which had been washed with 80% acetonitrile (ACN)–0.1% TFA and equilibrated with 0.1% aqueous TFA. After loading, the column was washed with 4 column volumes of 0.1% aqueous TFA, 4 column volumes of 10% ACN–0.1% TFA, and 4 column volumes of 30% ACN–0.1% TFA (to remove CSF). ComX pheromone was eluted with 3 column volumes of 60% ACN–0.1% TFA. The 60% ACN–0.1% TFA eluate was dried in a Speed-Vac to remove ACN–TFA, resuspended in 600 μl of minimal medium–50 μg of bovine serum albumin, and stored at 4°C. Serial twofold dilutions of the resuspended ComX pheromone were assayed for the ability to induce expression of a *srfA-lacZ* reporter in a strain unable to produce ComX pheromone (see below).

To assess the efficiency of ComX pheromone purification by this method, the recovery of a known amount of ComX pheromone added to conditioned medium from a  $\Delta comQ$  strain (which makes no ComX pheromone) was monitored. We found that  $\approx 50\%$  of added ComX pheromone was consistently recovered; the extent of recovery was not affected by the concentration of ComX pheromone (6 to 346 nM) (data not shown).

**Bioassay for ComX pheromone.** The activity of partially purified ComX pheromone was determined as described previously (24, 34). Briefly, resuspended ComX pheromone was added to JMS424 cells (35) at low density ( $OD_{600} = \approx 0.1$ ). JMS424 contains a *srfA-lacZ* fusion and is defective in ComX phero-

none production due to a null mutation in *comQ*. Each sample was incubated at 37°C for 70 min, and the β-galactosidase activity was determined (24). ComX pheromone activity is defined as (specific activity of sample) – (specific activity of indicator cells + fresh medium) and is presented as activity per milliliter of conditioned medium.

**Quantitative immunoblotting.** To prepare protein extracts for Western blotting, strains were grown in defined minimal medium, cells were harvested by centrifugation (Sorvall GS-4 rotor, 5,000  $\times g$ , 4°C), and cell pellets were stored at  $-80^\circ\text{C}$ . Cells were resuspended in ice-cold lysis solution (20 mM Tris-HCl, pH 8.0, 10% sucrose) and disrupted by sonication (Branson sonifier, three to four 30-s bursts, power setting 4, 50% duty cycle). The sonicate was centrifuged at 20,000  $\times g$  for 30 min at 4°C (Sorvall SS-34 rotor) to remove insoluble material and unbroken cells, and the supernatant of this spin was designated the total cell extract. Soluble protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard. Then 5 $\times$  sodium dodecyl sulfate (SDS) sample buffer (2) was added to a final concentration of 1 $\times$ , and protein samples were stored at  $-80^\circ\text{C}$ .

Protein samples were heated at 37°C for 5 min, vortexed, and briefly centrifuged before loading on sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to an Immobilon P polyvinylidene difluoride membrane (Millipore) using a Bio-Rad semidry transfer system. Membranes were blocked by incubation in 5% nonfat dry milk in Tris-buffered saline plus Tween 20 (TBST) for at least 1 h. The primary antibody was an affinity-purified (12) polyclonal rabbit antiserum raised against a glutathione *S*-transferase (GST)–ComQ (amino acids 183 to 299) fusion. Anti-rabbit immunoglobulin G (IgG)–alkaline phosphatase conjugate (Amersham) was used as the secondary antibody and was diluted 1:2,500 in TBST. Blots were developed using the ECF kit (Amersham), scanned on a Fluorimager 595 (Molecular Dynamics), and quantitated using ImageQuant 5.0 software (Molecular Dynamics). Images were processed using Adobe Photoshop 5.5.

## RESULTS AND DISCUSSION

**Overexpression of *comQ* and *comX* or *comX* alone alters the expression pattern of a quorum-responsive gene, *srfA*.** In wild-type *B. subtilis* cells, transcription of *srfA* is activated in re-

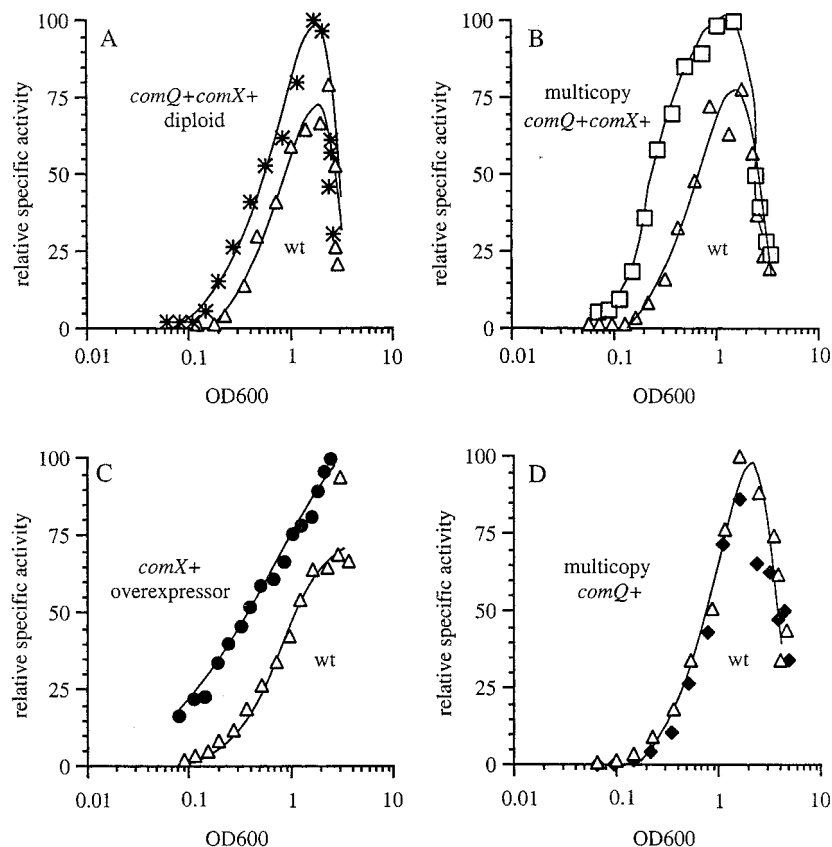


FIG. 2. Expression of *srfA-lacZ* in strains overexpressing *comQ* and *comX*. Strains were grown in defined minimal medium with appropriate antibiotics for plasmid selection and required amino acids. Samples were harvested at indicated culture densities ( $OD_{600}$ ) for determination of  $\beta$ -galactosidase specific activity. For each set of strains (panels A, B, C, and D), the maximal specific activity attained by the *comQ*<sup>+</sup> and/or *comX*<sup>+</sup> overexpressor was set at 100; all other data points were expressed relative to this value. All strains carry an *srfA-lacZ* transcriptional fusion (*srfA-lacZ*Ω682 *neo*) at the *amyE* chromosomal locus. (A) Stars, *comQ comX* diploid (KTB102); triangles, wild type (KTB121). (B) Squares, multicopy *comQ comX* (TMH326); triangles, wild type containing the cloning vector pHP13 (TMH327). (C) Solid circles, Pspac-hy-*comX* (KTB360); triangles, wild type (KTB346). KTB360 and KTB346 were grown in the presence of 1 mM IPTG for 2 to 3 generations prior to collection of samples for  $\beta$ -galactosidase activity determination. (D) Solid diamonds, multicopy *comQ* (KTB349); triangles, wild type with cloning vector pHP13 (TMH327).

sponse to increasing culture density (24). Transcription of *srfA* (as measured with an *srfA-lacZ* fusion) is low early in exponential growth and increases as cells approach stationary phase (Fig. 2A). Null mutations in *comQ* or *comX*, the two genes required for ComX pheromone production, cause reduced expression of *srfA* and a defect in transformability (24). We found that cells that overexpress both *comQ* and *comX* by twofold activated transcription of *srfA* approximately one-half generation earlier than wild-type cells (Fig. 2A). Cells that express *comQ* and *comX* on a *B. subtilis* multicopy plasmid (pTH2) induced transcription of *srfA* approximately 1 to 1.5 generations earlier than wild-type cells (Fig. 2B).

Overexpression of *comX* alone but not *comQ* alone was sufficient to cause an altered pattern of *srfA* expression. Cells that overexpress *comX* alone induced *srfA* transcription 1.5 to 2 generations earlier than the isogenic wild-type strain (Fig. 2C). In contrast, cells that overexpress *comQ* alone induced *srfA* transcription at the same cell density as wild-type cells (Fig. 2D).

Quantitative immunoblots confirmed that increasing gene dosage of *comQ* caused increased intracellular ComQ protein

concentration. Wild-type strains contained about 2,250 molecules of ComQ per cell (data not shown). The *comQ comX* diploid made approximately twofold more ComQ protein than wild-type cells (Fig. 3A), and strains carrying *comQ* alone or *comQ* and *comX* on the multicopy plasmid made approximately 10- to 15-fold more ComQ protein than wild-type cells containing the cloning vector (Fig. 3B and D). Cells overproducing *comX* alone had no increase in ComQ protein levels (Fig. 3C). We were not able to reliably detect ComX protein by immunoblotting with polyclonal antibodies to ComX.

**Measurement of ComX pheromone in culture supernatant.** We measured the amount of ComX pheromone activity in culture supernatant. Briefly, samples were taken from cells growing exponentially in defined minimal medium. Each sample was fractionated to separate ComX pheromone from other factors that influence *srfA* transcription (e.g., CSF) (34, 35). The partially purified ComX pheromone was then assayed for its ability to induce transcription of an *srfA-lacZ* reporter in a strain that does not produce ComX pheromone (see Materials and Methods). To assess the efficiency of ComX pheromone purification by this method, the recovery of a known amount of

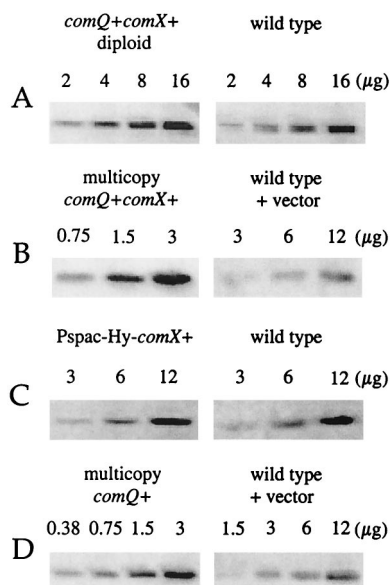


FIG. 3. ComQ protein levels in strains overexpressing *comQ* and/or *comX*. Strains were grown in defined minimal medium, and samples were harvested during exponential growth. ComQ protein was detected as described in Materials and Methods. Using purified ComQ protein, we estimate that there are approximately 2,200 molecules of ComQ per cell. Panels on the left show samples from an overproducer strain, and panels on the right show samples from the appropriate control. Serial twofold dilutions of protein samples were run to show the sensitivity and linearity of detection, and numbers above each lane indicate the amount of total protein run in that lane. Data presented are representative of three independent experiments. In all cases, samples for direct comparison were run on the same gel and subjected to the same blotting conditions. Apparent differences in the wild-type controls from panel to panel reflect different exposure times for the enhanced chemifluorescence. (A) *comQ comX* diploid (KTB345) and wild type (KTB344). (B) Multicopy *comQ comX* (TMH142) and wild type with the cloning vector pHP13 (TMH143). (C) Pspac-hy-*comX* (KTB360) and wild type (KTB346). (D) Multicopy *comQ* (KTB348) and wild type with the vector pHP13 (TMH143).

ComX pheromone added to conditioned medium from a  $\Delta comQ$  strain (which makes no ComX pheromone) was monitored. We found that  $\approx 50\%$  of added ComX pheromone was consistently recovered; the extent of recovery was not affected by the concentration of ComX pheromone added (6 to 346 nM) (data not shown). Results presented below are not adjusted for estimated losses during handling and are probably underestimates.

The amount of ComX pheromone in culture supernatant increased in parallel with cell number throughout growth (Fig. 4). Furthermore, production of ComX pheromone is not significantly affected by the ComP-ComA two-component regulatory system that is required for the cellular response to the pheromone (24). These findings are in contrast to several other quorum-sensing systems, in which accumulation of a critical concentration of a signaling molecule leads to a sudden increase in synthesis of the signaling molecule (7). In the analogous signaling systems in the gram-positive bacteria *Streptococcus pneumoniae* and *Staphylococcus aureus*, the two-component systems that are needed for response to the peptide pheromone also activate production of the pheromone (14, 28).

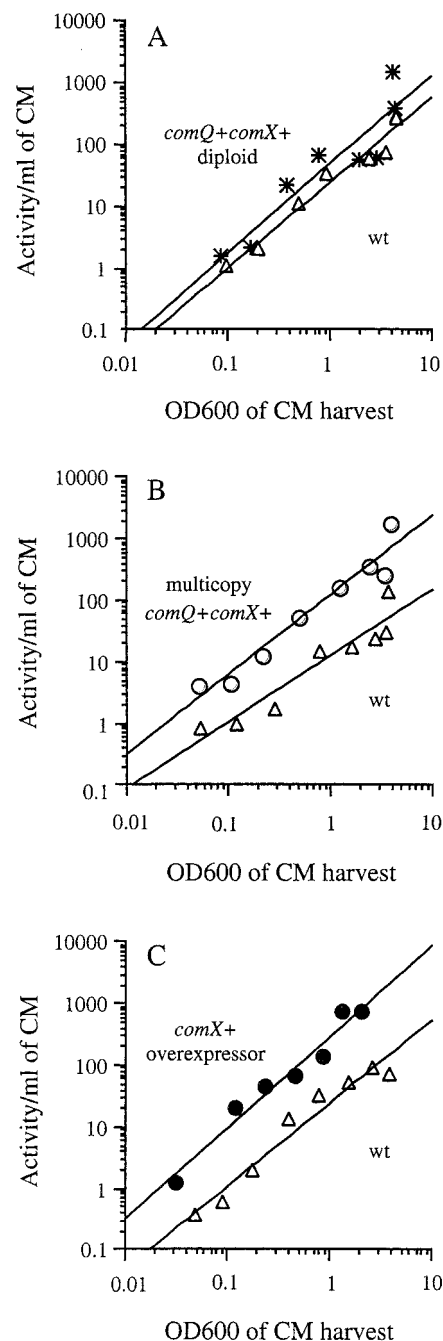


FIG. 4. ComX pheromone levels in strains overexpressing *comX*. Indicated strains were grown in defined minimal medium, and conditioned medium (CM) was harvested at various culture densities. Conditioned medium was fractionated to partially purify ComX pheromone, and ComX pheromone activity was assayed for the ability to induce expression of an *srfA-lacZ* fusion in a strain unable to produce its own ComX pheromone (JMS424). Graphs present the ComX pheromone activity in conditioned medium as a function of the culture density (optical density at 600 nm) at which the conditioned medium was harvested. (A) Triangles, wild type (KTB344); stars, *comQ comX* diploid (KTB345). (B) Triangles, wild-type (TMH143); open circles, multicopy *comQ comX* (TMH142). (C) Triangles, wild type (KTB346); solid circles, Pspac-hy-*comX* (*comX* overexpressor, KTB360). Pspac-hy-*comX* and the wild-type control were grown in the presence of 1 mM IPTG for 2 to 3 generations before the first conditioned medium sample was harvested.

We also measured the amount of ComX pheromone in conditioned medium from strains with extra copies of *comQ* and/or *comX*. These included strains with two copies of *comQ* *comX*, multiple copies of *comQ* and *comX*, and a strain overexpressing *comX* from a heterologous promoter. Strains that contained an extra copy of *comQ* and *comX* (*comQ* *comX* diploid) made approximately twice as much ComX pheromone as wild-type cells (Fig. 4A), whereas cells that expressed *comQ* and *comX* from the multicopy plasmid pTH2 made  $\approx 5$ - to 10-fold more ComX pheromone than wild-type cells (Fig. 4B). The *comX*-overexpressing strain produced approximately 10-fold more ComX pheromone than the isogenic wild-type strain (Fig. 4C).

These results indicate that the concentration of ComX pheromone in the extracellular milieu plays a major role in determining the timing of expression of quorum-responsive genes (*srfA*). The data also indicate that in wild-type cells, the expression of *comX* normally limits ComX pheromone production. ComQ levels, on the other hand, appear to be in excess of what is required for ComX pheromone production.

**Determinants of ComX pheromone activity.** To begin to identify the determinants of ComX pheromone activity in vivo, we performed alanine-scanning mutagenesis of the final 9 codons (DPITRQWGD) of *comX*. We tested each mutant allele for its ability to complement a *comX* null mutation in vivo. Complementation of the *comX* null mutation was judged by monitoring the expression of an *srfA-lacZ* transcriptional fusion.

The ComX alanine mutants fell into three groups based on the ability to complement the *comX* null mutation (Fig. 5). (i) *comXT50A*, *comXG54A*, and *comXD55A* appeared to be fully functional. In these mutants, the pattern of *srfA-lacZ* expression and accumulation was very similar to that in the wild type (Fig. 5D, 5H, and 5I). (ii) The *comXW53A* mutant, in which the tryptophan that is modified during the production of ComX pheromone is eliminated, was completely nonfunctional. The pattern of *srfA-lacZ* expression in this mutant was indistinguishable from that of the *comX* null (Fig. 5G). (iii) The remaining alleles were partly functional, with apparent quantitative differences. In strains carrying the *comXP48A* or *comXR51A* allele, induction of *srfA-lacZ* was delayed by 2 generations or more, and accumulation peaked at a level approximately 20 to 40% of that in wild-type cells (Fig. 5B and 5E). In strains carrying the *comXD47A*, *comXI49A*, and *comXQ52A* alleles, induction of *srfA-lacZ* was delayed approximately 1 generation and accumulation peaked at 50 to 60% of the wild-type level (Fig. 5A, 5C, and 5F).

The finding that the *comXT50A*, *comXG54A*, and *comXD55A* alleles fully complement the *comX* null mutation indicates that the side chains of the amino acid products are not essential for the activity of ComX pheromone. These residues are unlikely to make significant contact with any protein(s) involved in production of ComX pheromone, nor are they likely to be involved in the ComX pheromone-Comp receptor interaction. Interestingly, the *comXG54A* allele seems to activate *srfA-lacZ* expression slightly better than wild-type *comX* (Fig. 5). This suggests that replacing the glycine at this position with alanine may create a new, positive contact with a protein involved in production of ComX pheromone or with Comp.

The results of the alanine-scanning mutagenesis also highlight the importance of the modification on ComX pheromone for activity. An unmodified, 10-amino-acid peptide (ADPITRQWGD) is unable to activate transcription of *srfA* when added exogenously to cells (24). Converting the tryptophan residue that is the site of modification to alanine almost certainly renders the ComX pheromone precursor unmodifiable. We have not been able to determine if cells expressing the *comXW53A* allele can export an unmodified ComX pheromone precursor.

The amino acids at positions 47 to 49 (corresponding to *comXD47*, *comXP48*, and *comXI49*) and 51 and 52 (*comXR51* and *comXQ52*) of ComX are good candidates to make contacts with the protein(s) involved in ComX pheromone production or with Comp. The experiments described here do not allow us to determine whether production of or response to ComX pheromone is affected in strains carrying these mutations. We hope to be able to isolate production intermediates from strains expressing *comX* mutant alleles to help us further define which residues are important for production, activity, or both.

**Putative isoprenoid binding domain of ComQ is required for ComQ function in vivo.** *comQ* was originally identified as a regulatory gene that affected the development of genetic competence (40). *comQ* null mutants are defective in ComX pheromone production (24) and have decreased expression of *srfA* (40). Recent homology searches identified IdsA, the bifunctional short-chain isoprenyl diphosphate synthase of *Methanobacterium thermoautotrophicum*, as the protein most similar to ComQ (23, 38). Isoprenyl diphosphate synthases catalyze the formation of long-chain isoprenoids through the condensation of isopentenylpyrophosphate (IPP) and allylic diphosphates (the simplest of which is dimethylallyl pyrophosphate) (8).

The similarity of ComQ to IdsA is intriguing because the mass of the modification (206 Da, assuming the tryptophan side chain is intact) (24) and the hydrophobicity that it imparts on ComX pheromone are consistent with farnesylation. Additionally, the gene for *comQ* is found directly upstream of *comX* in the chromosome as part of a peptide signaling cassette (16, 18, 23, 41). The genes involved in production of autoinducing peptide (AIP) in *S. aureus* are also contained in a peptide signaling cassette. AIP is a modified peptide, and the gene required for processing and modification (*agrB*) is located immediately upstream of the gene encoding the AIP precursor (*agrD*) in the *S. aureus* chromosome (17, 28). Although there is no obvious sequence similarity between ComQ and AgrB, if conserved genetic organization suggests parallel function, then ComQ is likely to modify and/or process ComX pheromone.

A partial alignment of ComQ with IdsA and other IPPases shows that a region known to be important for IPPase activity (4) is especially well conserved in ComQ (region II) (Fig. 6). Region II contains a highly conserved, aspartate-rich domain (1) which is thought to interact with one of the two isoprenoid moieties that are condensed by IPPases (4). These aspartate residues are required for the in vitro activity of IPPases (25, 36).

To determine if the conserved aspartates in region II of ComQ are required for ComQ function, two aspartic acid residues (ComQD67 and ComQD71) were converted to glu-

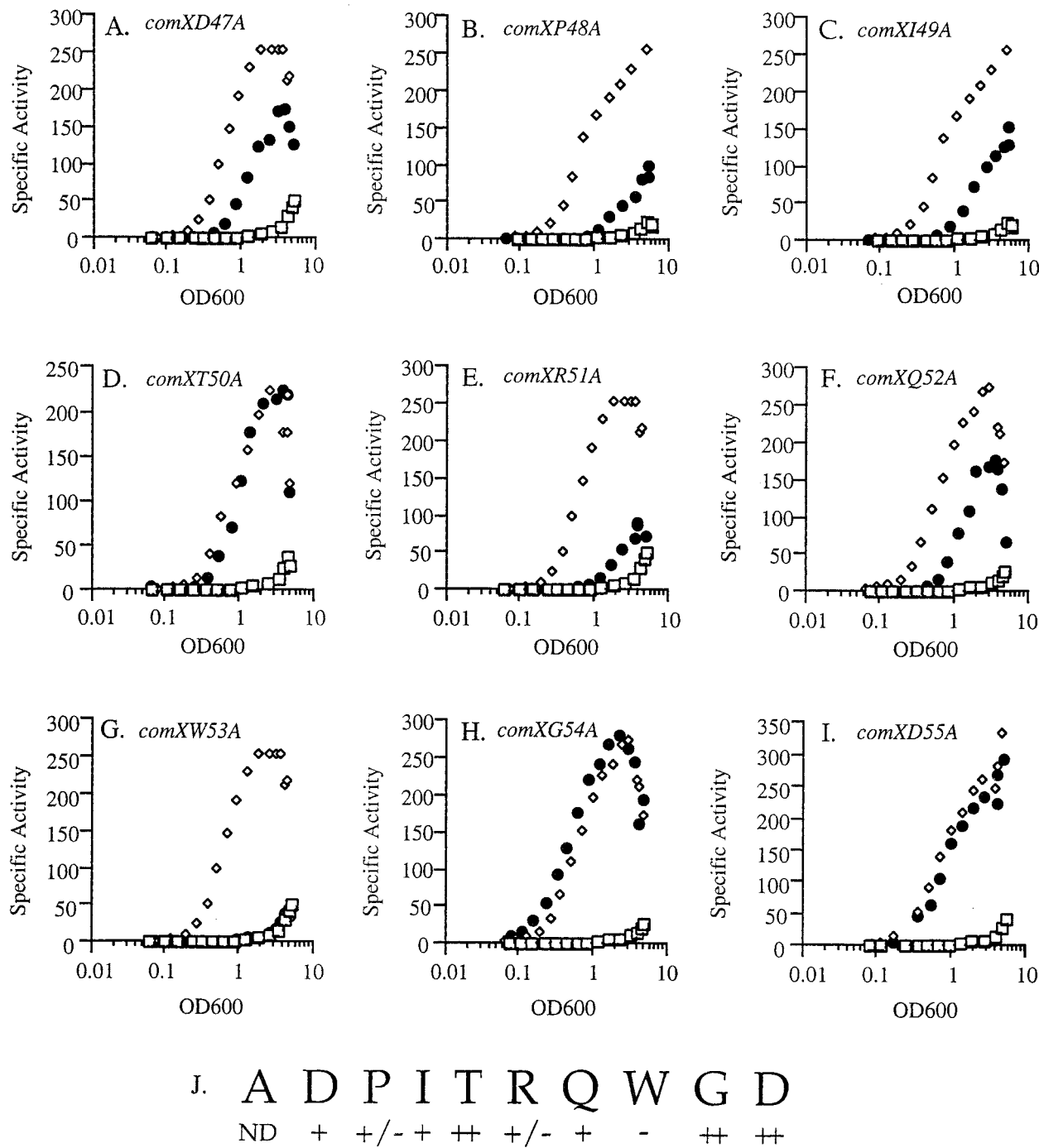


FIG. 5. Expression of *srfA-lacZ* in ComX alanine mutants. Functions of the *comX* mutants were judged by monitoring expression of an *srfA-lacZ* transcriptional fusion. Strains were grown in defined minimal medium, and samples were taken every 30 min for determination of  $\beta$ -galactosidase specific activity. Data presented are representative of three independent experiments. In panels A to I, KTB172 ( $\Delta comX$ ) is represented by squares and KTB280 ( $comX^+ \Delta comX$ ) is represented by diamonds. Strains containing mutant alleles are represented by solid circles. (A) KTB310 (*comXD47A*  $\Delta comX$ ). (B) KTB300 (*comXP48A*  $\Delta comX$ ). (C) KTB302 (*comXI49A*  $\Delta comX$ ). (D) KTB363 (*comXT50A*  $\Delta comX$ ). (E) KTB288 (*comXR51A*  $\Delta comX$ ). (F) KTB304 (*comXQ52A*  $\Delta comX$ ). (G) KTB308 (*comXW53A*  $\Delta comX$ ). (H) KTB282 (*comXG54A*  $\Delta comX$ ). (I) KTB306 (*comXD55A*  $\Delta comX$ ). (J) Summary of activity of the alanine mutants. Symbols: ++, functional; +, mostly functional; +/—, partially functional; —, nonfunctional. ND, not done.

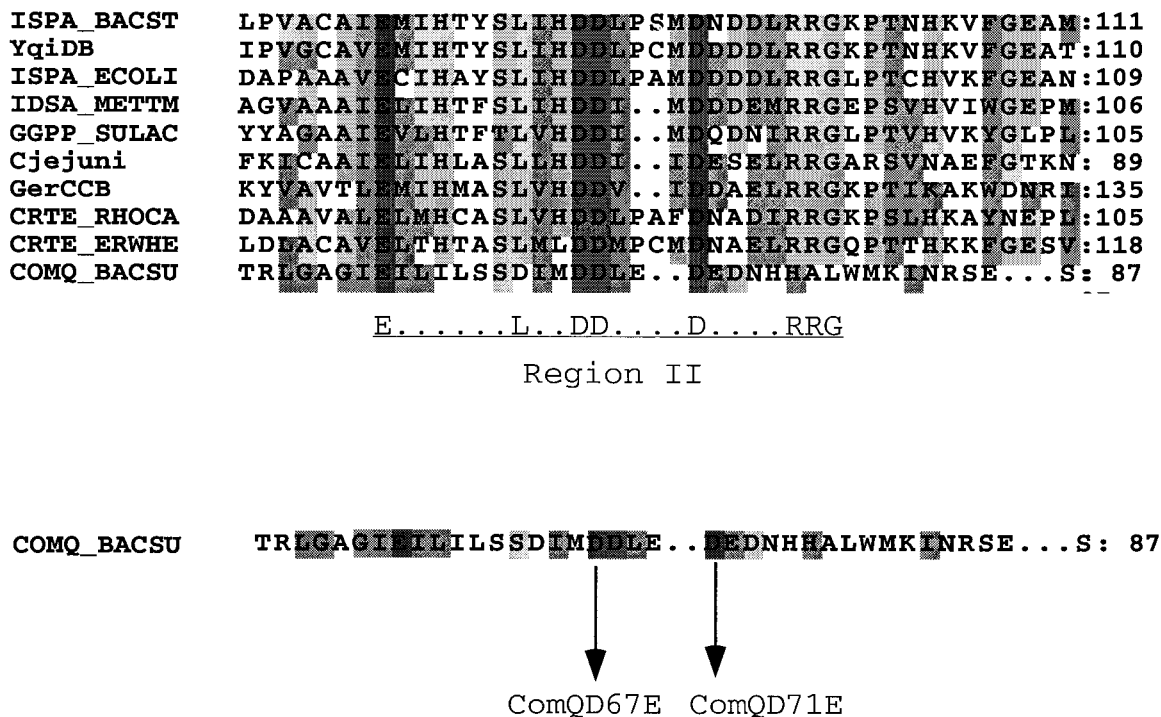


FIG. 6. Alignment of region II of IPPases with part of ComQ. ComQ and IPPases were aligned using ClustalW. Conserved residues are shaded light gray, similar residues are gray, and residues identical in ComQ and all IPPases are shaded dark gray. ISPA\_BACST, IspA from *Bacillus stearothermophilus*; YqiDB, YqiD from *B. subtilis* (putative synthase); ISPA\_ECOLI, IspA from *E. coli*; IDSA\_METTM, IdsA from *Methanobacterium thermoautotrophicum*; GGPP\_SULAC, geranylgeranyl pyrophosphate synthetase from *Sulfolobus acidocaldarius*; Cjejuni, putative polyprenyl synthase from *Campylobacter jejuni*; GerCCB, GerCC from *B. subtilis*; CRTE\_RHOCA, CrtE from *Rhodobacter capsulatus*; CRTE\_ERWHE, CrtE from *Erwinia herbicola*; COMQ\_BACSU, ComQ from *B. subtilis*. Conserved region II is labeled as described (4), and the consensus sequence is listed. The two aspartates converted to glutamate by site-directed mutagenesis (D67E and D71E) are indicated in the lower diagram.

tamic acids by oligonucleotide-directed mutagenesis (Fig. 6). Each mutant *comQ* allele was tested for its ability to complement a *comQ* null mutation when expressed from the native *comQ* promoter on a *B. subtilis* multicopy plasmid (pHP13). The readout for ComQ function was the induction of an *srfA-lacZ* fusion, which is indicative of ComX pheromone production.

Neither the *comQD67E* mutant nor the *comQD71E* mutant allele was capable of complementing the *comQ* null mutation. *srfA-lacZ* expression in these mutants resembled that of the *comQ* null mutant (Fig. 7A). The ComQD67E and ComQD71E proteins are present at levels similar to or higher than the ComQ levels in wild-type cells (Fig. 7B). Thus, the *comQD67E* and *comQD71E* mutations eliminate ComQ function in ComX pheromone production.

Though ComQ shares conserved regions with isoprenyl synthases, it is unlikely that ComQ is the dedicated isoprenoid synthase in *B. subtilis*. *B. subtilis* encodes a well-characterized, two-subunit heptaprenyl diphosphate synthase (encoded by *hepS* and *hepT*), involved in the synthesis of menaquinone (42, 43), and a probable undecaprenyl diphosphate synthase (*uppS*) (20). We hypothesize that the aspartate-rich domain in region II of ComQ binds an existing modification substrate, perhaps farnesyl diphosphate generated by one of these enzymes, and then attaches it to the tryptophan of ComX as part of the pheromone production pathway.

Alignment of ComQ sequences from *B. subtilis* RS-B-1 (37), *B. subtilis* 168, and *B. mojavensis* RH-O-1 (37) showed that these proteins are 39% identical over their entire length (data not shown). The aspartate-rich domain in region II, however, is 86% identical among these three strains (Fig. 7C). The predicted ComX pheromones from strains RS-B-1 and RH-O-1 are nearly identical (37) but show no sequence identity with ComX pheromone from strain 168 except for the tryptophan that is the site of modification (37). The modification on ComX pheromone is likely to be the same in these strains, and the conserved aspartate-rich domain of ComQ is probably involved in attaching the modification to the conserved tryptophan.

**Specificity determinants in peptide signaling.** Recent work has shown that there is specificity between ComX pheromones and their cognate receptors in *B. subtilis*, *B. natto*, and *B. mojavensis* (37, 38). If the ComX pheromone modification is conserved, then this modification cannot serve as a determinant of specificity. Rather, the hydrophobicity of the modification may serve to increase the local concentration of ComX pheromone near the membrane. Once membrane associated, the ComX peptide backbone could make the specific contacts with the ComP receptor required for signal transduction. Farnesylation of yeast a mating factor has been shown to be important for membrane association (3, 5) and may be directly involved in the presentation of a factor to its receptor (6).

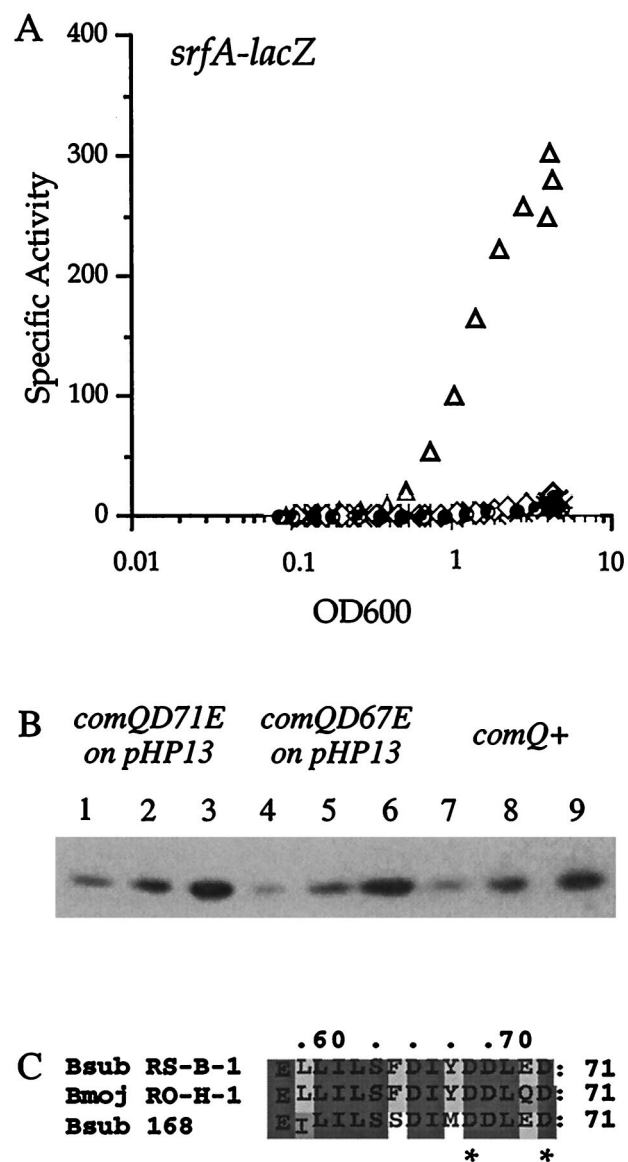


FIG. 7. Functional analysis of *comQD67E* and *comQD71E* mutant alleles. (A) Functions of the *comQ* mutant alleles were assayed by measuring expression of an *srfA-lacZ* fusion. Cells were grown in defined minimal medium, and samples were taken for determination of  $\beta$ -galactosidase specific activity. All strains carry an *srfA-lacZ* transcriptional fusion (*srfA-lacZ* $\Delta$ 682 *neo*) integrated at the *amyE* chromosomal locus. Diamonds, *comQD67E* strain KTB216 (*comQ::spc* pKB28 [*comQD67E* in pHP13] *thrC::mIs*); stars, *comQD71E* strain KTB217 (*comQ::spc* pKB29 [*comQD71E* in pHP13] *thrC::mIs*); solid circles, *comQ* null strain KTB122 (*comQ::spc* *thrC::mIs*); triangles, *comQ*<sup>+</sup> strain KTB131 (*comQ::spc* *thrC::comQ*<sup>+</sup> *mIs*). (B) Immunoblot of total cellular protein probed with antibodies against ComQ. Lanes 1 to 3, KTB217 (*comQD71E* on pHP13); lanes 4 to 6, KTB216 (*comQD67E* on pHP13); lanes 7 to 9, KTB131 (*thrC::comQ*<sup>+</sup>). Total protein loaded was 1  $\mu$ g (lanes 1, 4, and 7), 2  $\mu$ g (lanes 2, 5, and 8), and 4  $\mu$ g (lanes 3, 6, and 9). (C) Alignment of the ComQ putative isoprenoid binding region from *B. subtilis* (Bsub) RS-B-1, *B. mojavensis* (Bmoj) RO-H-1, and *B. subtilis* 168. Sequences were aligned using ClustalW and shaded using MacBoxShade. Conserved residues are shaded light gray, similar residues are gray, and residues identical in all three sequences are shaded dark gray. The aspartates shown to be required for ComQ function in *B. subtilis* 168 are indicated with asterisks.

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